applying our ED protocol to different number of subunits (SUs). Our simulations showed that the movement of a single SU is not sufficient to open the activation gate. But by moving three SUs by ED simulations, the activation gate opened to the same extent as in the four SUs ED simulation protocol. These finding is in line with fluorescence detection studies, which showed that the SUs act cooperatively during gating (Blunck et al. 2008).

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Gating Motions of KirBac1.1 Cytoplasmic Domain with Respect to Transmembrane Domain Revealed by FRET

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KirBac1.1 is a bacterial inward rectifier potassium (Kir) channel, which, contrary to its eukaryotic homologues, is strongly inhibited by phosphatidylinositol-4,5-bisphosphate (PIP2). The most recent crystal structures of eukaryotic Kir2.2 in complex with PIP2 indicate that the TM-CD linker forms a short a-helix in the presence of PIP2. As a result, the cytoplasmic domain of Kir2.2 moves about 6 angstrom towards the membrane surface. However, the 'KKR' motif in the TM-CD linker of Kir2.2, which directly interacts with PIP2, is absent in KirBac1.1 and the question arises: how does KirBac1.1 cytoplasmic domain move in response to PIP2 inhibition? In the present work, we have made KirBac1.1 tandem tetramer constructs and purified KirBac1.1 tandem proteins successfully. Reconstituted liposome flux assays indicate that the KirBac1.1 tandem protein remains functional, and retains sensitivity to PIP2 inhibition. We introduced two cysteine into the KirBac1.1 tandem tetramer, one at the extracellular loop of subunit 1 (G85 or T120) and one in the cytoplasmic domain of subunit 2 (A273). We labeled these cysteines with a FRET dye pair (Alexa-Fluor 488 and DABCYL) and measured FRET efficiencies in protein samples reconstituted into liposomes, in the absence and presence of PIP2. Our results indicate that the KirBac1.1 cytoplasmic domain moves ~2-3 angstrom away from the transmembrane domain in the presence of PIP2 - opposite the direction suggested from eukaryotic Kir2.2 crystal structures in the presence and absence of PIP2. Reversed PIP2-dependent motions of the cytoplasmic domain with respect to the transmembrane domain between prokaryotic and eukaryotic Kir channels may explain their differential response to PIP2 modulation.

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Simulations of the Helix Bundle Crossing Gate Opening in Kir Channels

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Inwardly rectifying K^+ (Kir) channels are gated by the phospholipid PIP_2. Along the ion permeation pathway, three relatively narrow regions (the selectivity filter -SF, the inner helix bundle crossing (HBC), and the intracellular G-loop) may serve as gates to control ion permeation. A crystal structure of a Kir3.1 chimera [Nishida et al., 2007] captured the cytosolic G-loop gate in ''closed/constricted'' or ''open/dilated'' conformations. 100 ns Molecular Dynamics (MD) simulations studying the PIP_2-driven Kir channel activation of the Kir3.1 chimera led us to propose a molecular mechanism of the G-loop gate opening [Meng et al., 2012]. However, opening of the HBC gate was not observed throughout this simulation. Mutagenesis and single-channel recording studies in our lab showed that a proline mutation on the inner helix of the Kir3.4 channel dramatically increased the open probability of the channel [Jin et al., 2002]. We introduced the corresponding M170P mutation on the Kir3.1 chimera structure and ran 100 ns long simulations of four mutant channel systems: dilated and constricted M170P Kir3.1 chimera in the presence (holo) and absence (apo) of PIP_2, using the GROMACS program [Hess et al., 2008]. Three potassium ions present in the SF passed through the HBC gate in the system of the holo dilated M170P Kir3.1 chimera within the 100 ns simulation time. Minimal distance measurements indicated that the HBC gate was able to open only when PIP_2 was present and the G-loop gate was stabilized in the open state. Principal component analysis revealed coupled conformational changes in the Slide helix, DE- and LM-loops, possibly related to the opening of the HBC gate. Moreover, unique residue interactions within the transmembrane domains were observed in the dilated holo system. Predictions of these models are being tested experimentally.

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Native Currents in Hepatocytes with Characteristic Properties of Kir2 Channels

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We investigated the electrophysiological properties of parenchymal liver cells (hepatocytes) by using the perforated patch-clamp technique with Amphotericin B. We found that dissociated mouse hepatocytes exhibited native currents with characteristic properties of inward rectifier potassium (Kir) channels, subfamily 2, which has not been previously reported. Currents were constitutively active and stable for longer than 30 min during recording, with a current density of -16.2 ± 1.7 pA/pF ($V_m = -114$ mV, current at $[K^+]_{out} = 5$ mM minus current at $[K^+]_{out} = 0$ mM, n=14 cells). Currents exhibited strong and ''steep'' inward rectification, with essentially no outward current at voltages more positive than -20 mV, as typically seen with Kir2 channels. The reversal potential approximated the predicted E_K (-81.8 ± 0.4 *vs.* -84.4 mV, $[K^+]_{in} = 134$ mM and $[K^+]_{out} = 5$ mM). Varying $[K^+]_{out}$ over a range of 5 to 144 mM showed that E_{rev} was strongly dependent on $[K^+]_{out}$, with a Nernstian slope of 58.8 mV/decade, demonstrating that the currents are highly selective for K^+. Currents were fully blocked by external Ba^{2+}, with $K_{1/2} = 2.7 \pm 0.2$ μM ($V_m = -94$ mV, $[K^+]_{out} = 60$ mM, n=6 cells). This $K_{1/2}$ value is in close agreement with published data on Ba^{2+} block of homomeric Kir2.1 channels (Liu GX et al. *J Physiol* 2001). The currents were not significantly inhibited by acidification of the bath solution or pipette solution, which argues against a contribution of pH-sensitive Kir2 subunits such as Kir2.2 or Kir2.3. We thus hypothesize that the molecular identity of the observed currents is homomeric Kir2.1. Additional experiments are underway to test this hypothesis and to elucidate the physiological role of native Kir2 channels in hepatocytes.

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Reduced PIP2 Binding to KCNJ2 (M307I) Channels is Linked to Type 1 Andersen-Tawil Syndrome

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Background: Inward rectifier potassium channels play a key role in setting and maintaining the resting membrane potential and regulating excitability in various tissues. Kir2.x subfamily members mediate the cardiac inward rectifier potassium current (IK1). KCNJ2 encodes Kir2.1, the pore-forming alpha subunit responsible for cardiac IK1, and the mutations in this gene are associated with type 1 Andersen-Tawil Syndrome (ATS1). A Kir2.1 missense mutation, M307I, has been identified in a Korean family with ATS1. We found that the ATS1-associated M307I mutation is a loss-of-function mutation in KCNJ2 that mediates a dominant-negative effect on wild-type (WT) channels. M307I is located in the intracellular C-terminal domain in a region known to be associated with putative phosphatidylinositol 4,5-bisphosphate (PIP2) binding and channel trafficking. Here we explored the mechanisms underlying the dominant-negative effect of the mutation.

Methods and Results: Human Kir2.1 was subcloned into pFlag-CMV vector and pFlag-Kir2.1-M307I was generated by site-direct mutagenesis. The Flag-Kir2.1-WT and Flag-Kir2.1-M307I were expressed in HEK293 cells and affinity purified. PIP2-binding was assessed using a Lipid-bead-protein pull-down assay with cell lysate and Protein-lipid overlay assay with purified proteins. The electrophysiological data showed that the M307I mutant channel significantly reduces whole cell current densities when co-expressed with Kir2.1-WT channels. Immunofluorescence (IF) staining assays reveal that M307I channels exhibit normal membrane trafficking. PIP2 binding assays show that Flag-Kir2.1-M307I channels exhibit dramatically decreased binding to PIP2 compared to WT channels.

Conclusions: M307I is an ATS1-associated, loss-of-function missense mutation in KCNJ2 that mediates a dominant-negative effect on WT channels by reducing PIP2 binding to the channel.

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Targeting of $K_{ir}2.1$ and Downregulation of Inward Rectifier K^+ Current by miR-212

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Downregulation of inwardly rectifying K^+ channels contributes to an increased risk of cardiac arrhythmia in heart failure and to impaired cerebral arterial dilation in chronic alcohol consumption. The downregulation mechanism is unknown, although post-transcriptional regulation of gene expression by microRNAs is a strong possibility that has not been fully investigated. miR-212 is markedly augmented in heart failure and in chronic alcoholism, and is predicted by bioinformatic algorithms to target $K_{ir}2.1$, the predominant inward rectifier K^+ channel expressed in heart and arterial smooth muscle. We developed a fluorescence-based assay for identifying microRNA targets, using mCherry red

fluorescent protein to report target sequence expression and enhanced green fluorescent protein for microRNA expression. Using this assay, we demonstrated a functional target for miR-212 in the 3′ untranslated region of $K_{ir}2.1$. Red/green fluorescence intensity ratio was significantly lower in miR-212-expressing HEK293 cells compared to non-targeting control (miR-212 0.72 ± 0.024 (mean ± sem), n = 550; control 1.21 ± 0.025, n = 731; $p<.001$, log transformed data). The effect of miR-212 was attenuated by mutating the predicted target site (% inhibition 58.0 ± 14.51, n = 3 wild-type; 22.7 ± 1.25, n = 3 mutant). Expression of miR-212 downregulated endogenous $K_{ir}2.1$ protein in HeLa cells, as shown by quantitative western blot of membrane extracts (band intensity *vs* Na^+/K^+-ATPase loading control: miR-212 0.0647 ± 0.0047; non-targeting control 0.0895 ± 0.0045; n=3, $p<.05$). Endogenous inward rectifier K^+ current in HeLa cells was isolated by extracellular application of 100 μM Ba^{2+} during whole-cell patch-clamp recording. Ba^{2+}-sensitive current density was significantly smaller in miR-212-transfected (n=13) *vs* control-transfected cells (n=8); $p<.01$. In conclusion, downregulation of inwardly rectifying K^+ current and $K_{ir}2.1$ expression in heart failure and alcoholic cerebrovascular dysfunction may be functionally linked to upregulation of miR-212.

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Dissecting Gating Rules of GIRK Channels: Role of PIP2 in Ethanol-Dependent Activation

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G protein-gated inwardly rectifying potassium (GIRK) channels are implicated in alcohol abuse and addiction. We discovered a discrete alcohol-binding pocket in the channel mediating ethanol-dependent activation. Here, we investigated the role of G proteins and PIP_2 in ethanol-dependent gating. We engineered GIRK2 with single, modifiable cysteine at L257 in alcohol pocket and found that alcohol-like methanthiosulfonate (MTS) reagents activate GIRK2-L257C, similar to ethanol-dependent activation. We assessed role of G proteins in alcohol activation by either increasing levels of Gβγ (+Gβ1γ2) or decreasing Gβγ through chelation with membrane bound Phosducin (+mPhos). Neither Gβ1γ2 nor mPhos altered the rate of MTS-HE-mediated GIRK2-L257C activation. For comparison, we examined GIRK2-L344C, a key site for Gβγ activation that is inhibited by MTS modification. In contrast to L257C, rate of MTS modification showed a clear dependence on Gβγ levels. These results suggest that alcohol activation of GIRK channel is independent of G proteins. To investigate the role of PIP_2, we used voltage-sensitive phosphatase DR-VSP to transiently deplete PIP_2 in the membrane. Activation of DR-VSP completely reversed MTS-HE activated current of GIRK2-L257C. Furthermore, MTS-HE treatment significantly slowed the rate of GIRK2-L257C current inhibition following DR-VSP activation, suggesting an increase in apparent affinity for PIP_2 and GIRK2-L257C channels modified by MTS-HE. Lastly, we examined the role of PIP_2 on alcohol-dependent activation of wild-type GIRK2. Addition of propanol significantly slowed the rate of wild-type GIRK2 current inhibition following PIP_2 depletion. Taken together, these data demonstrate that alcohol-dependent activation of GIRK involves an increase in apparent affinity for PIP_2, with little influence of Gβγ subunits. The fundamental dichotomy between alcohol and Gβγ arises from distinct gating mechanisms converging on PIP_2-dependent opening, revealing novel pathways for antagonizing alcohol's effects on ion channels.

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Unique PIP2 Sensitivity at a Putative PKC Site in GIRK2 (Kir3.2)

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G-protein activated inward rectifier potassium channels (GIRKs) exhibit sensitivity to a diverse range of modulators, including G-proteins, sodium, PIP_2, and phosphorylation by PKA and PKC. The residue Ser-196 in the GIRK2 subunit is implicated in PKC sensitivity in the homologous GIRK1 and GIRK4 subunits. It is located distal to the helix bundle crossing, and is situated to interact with both Phe-192 of the bundle crossing and Thr-317 of the G-loop gate. In the background of the highly active homomeric GIRK2 mutant E152D, we mutated Ser-196 to Ala and tested its PIP_2 dependence, using the voltage-sensitive PIP phosphatase Ci-VSP. The S196A mutant was inhibited normally by activation of Ci-VSP, but upon recovery it displayed a unique behavior. Instead of a monophasic recovery, the S196A mutant exhibited a characteristic inhibition following recovery, which was not observed in the homomeric mutant alone. In addition, the S196A mutant current recovery depended on the initial level of PIP_2 depletion. Mutant channels S196E and S196Q did not reproduce the unique pattern of S196A. Using the G_q-coupled hM1 assay, we tested the muscarinic sensitivity of S196A vs. homomeric mutant. While the homomeric mutant was inhibited normally, the S196A channel did not show appreciable inhibition. Taken together, these results indicate that the S196A mutant exhibits unique PIP_2 sensitivity. Given the critical location of S196 to the channel gates we are pursuing the structural mechanism that could explain the unique behavior of the Ala mutant.

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HDAC Inhibitors Affect Sulfonylurea Receptor Subunit MRNA Expression in Atrial-Derived Hl-1 Cells but not Pancreatic Beta Cell-Derived MIN6 Cells

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K_{ATP} channels are expressed in many types of excitable cells where they typically act as key sensors of cell metabolism. All K_{ATP} channels share the same architecture—a K^+ channel pore (Kir6.1 or Kir6.2) combines with a sulfonylurea receptor (SUR1, SUR2A or SUR2B) to form a functional channel. Importantly, K_{ATP} channels composition is tissue specific. SUR1 and Kir6.2 make up the channel in atrial cardiomyocytes and pancreatic beta cell, while SUR2A combines with Kir6.2 to form ventricular myocyte K_{ATP}. Tissue specific heterogeneity appears to be driven principally by differential subunit transcription, but the mechanisms that determine when and where specific K_{ATP} channels are expressed are poorly understood. In this study, we have employed both cardiac- (HL-1) and pancreatic beta cell- (MIN6) derived cell lines to explore the mechanisms that control SURx gene expression. In both HL-1 and MIN6 cells we find that SUR1 expression is significantly greater than SUR2. When cells are treated for 72 hours with trichostatin A (a general inhibitor of histone deacetylases or HDACs), there is a significant increase in SUR2 subunit expression in HL-1 cells, but no apparent change in SUR2 expression in MIN6 cells. This result indicates that in the absence of HDAC activity, the transcriptional machinery to drive SUR2 gene expression is available in HL-1, but not in MIN6 cells. From this data, we conclude that both the SURx subunit transcriptional profile and the mechanisms that determine that profile are tissue specific.

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Lessons from KATP Channels with Diabetogenic Mutations in Sulfonylurea Receptor 1 (SUR1)

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Numerous mutations have been identified in SUR1 (ABCC8) subunit of the neuroendocrine type KATP channel in subjects with neonatal diabetes, neonatal diabetes plus epilepsy and/or other neurological features, maturity-onset diabetes of young, and later-onset diabetes. Patch-clamping, single-channel kinetics analysis, affinity photolabeling and molecular modeling were used to clarify how diabetogenic mutations in different parts of SUR1 affect open probability and sulfonylurea inhibition of SUR1/Kir6.2 KATP channel. Essentially all tested diabetogenic mutations in the canonical TMD1-NBD1-TMD2-NBD2 ABC exporter core of SUR1 hyperactivated KATP by stabilizing the stimulatory Mg-nucleotide bound (outward facing) state of SUR1 without affecting the intrinsic gating of KATP channel or its sensitivity to inhibitory nucleotides. Hyperstimulated mutant channels showed attenuated sulfonylurea inhibition in the presence, but not the absence, of stimulatory MgATP/ADP, indicating that KATP with SUR1 in the inward facing state has the lowest Kd for sulfonylureas. Diabetogenic mutations in the non-canonical TMD0-L0 part of SUR1 hyperactivated KATP by destabilizing its long-lived closed state with the highest affinity to inhibitory ATP or by strengthening the functional coupling between the MgATP/ADP-bound SUR1 core and the active (burst) state of the Kir pore. Mutations destabilizing the long-lived closed state compromised sulfonylurea inhibition of KATP in the absence of nucleotides but not the drug-induced release of stimulatory nucleotides. The findings support the mechanistic model (FEBS Letters, 585:3555-9) in which the TMD0-L0 module couples the SUR1 core with the KATP pore, define the most common ABCC8-associated mechanisms of KATP hyperactivity, and largely explain why the majority of diabetic subjects with mutant SUR1 require body-weight normalized doses of sulfonylureas exceeding those recommended by the FDA for treatment of common type 2 diabetes.

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A Single Point Mutation in the Distal C-Terminal of the Pore Forming Kir6.1 Subunit Modifies ATP-Sensitive Potassium (K_{ATP}) Channel Regulation

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